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EPIGALLOCATECHIN-3-GALLATE IS A POTENT NATURAL INHIBITOR OF FATTY ACID SYNTHASE IN INTACT CELLS AND SELECTIVELY INDUCES APOPTOSIS IN PROSTATE CANCER CELLS

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Chemical inhibitors of fatty acid synthase (FAS) inhibit growth and induce apoptosis in several cancer cell lines *in vitro* and in tumor xenografts *in vivo*. Recently the green tea component epigallocatechin-3-gallate (EGCG) was shown to act as a natural inhibitor of FAS in chicken liver extracts. Here we investigated whether EGCG inhibits FAS activity in cultured prostate cancer cells and how this inhibition affects endogenous lipid synthesis, cell proliferation and cell viability. The high levels of FAS activity in LNCaP cells were dose-dependently inhibited by EGCG and this inhibition was paralleled by decreased endogenous lipid synthesis, inhibition of cell growth and induction of apoptosis. In contrast, epicatechin (EC), another closely related green tea polyphenolic compound, which does not inhibit FAS, had no effect on LNCaP cell growth or viability. Treatment of nonmalignant cells with low levels of FAS activity (fibroblasts) with EGCG led to a decrease in growth rate but not to induction of apoptosis. These data indicate that EGCG inhibits FAS activity as efficiently as presently known synthetic inhibitors and selectively causes apoptosis in LNCaP cells but not in nontumoral fibroblasts. These findings establish EGCG as a potent natural inhibitor of FAS in intact cells and strengthen the molecular basis for the use of EGCG as a chemopreventive and therapeutic antineoplastic agent.

Key words: *apoptosis; EGCG; fatty acid synthase; prostate cancer*

Tea, the most consumed beverage worldwide next to water, is known to contain high amounts of polyphenolic compounds that may account for up to 40% of the extractable solids.[1–3] About 78% of the consumed tea is black tea, a popular drink in many Western countries, which contains mainly oxidized polyphenols, whereas 20% is green tea, which is primarily consumed in China and Japan and contains mostly nonoxidized polyphenols, more particularly catechins.[1,3] The major polyphenolic compound of green tea is epigallocatechin-3-gallate (EGCG), whereas other related catechins such as epicatechin (EC), epigallocatechin (EGC) and epicatechin-3-gallate (ECG) are present in green tea at lower levels.[1,3]

During the last decades, numerous epidemiological studies have shown that consumption of green tea is inversely correlated with the risk of developing various cancers (including cancer of the breast, prostate, stomach, esophagus, colon and pancreas).[2–6]

Furthermore, EGCG inhibits proliferation and induces apoptosis of several tumor cell lines *in vitro*[7–9] and decreases the size of tumors in mice and rats.[3,10–12] With regard to this cancer-preventive effect of EGCG, several molecules have been proposed as a target for green tea catechins, such as urokinase, matrix metalloproteinases, vascular endothelial growth factor (VEGF), tyrosine kinase receptors and cell cycle regulators.[3,10,13–15] However, the precise mechanisms by which EGCG exerts its anti-carcinogenic activity are still not fully understood.

Recently, EGCG was shown to act as a potent inhibitor of fatty acid synthase (FAS) in chicken liver extracts.[16] FAS is a key metabolic enzyme catalyzing the synthesis of long chain fatty acids from 2-carbon precursors.[17] In most human tissues, expression of FAS is low, but it is significantly elevated in a variety of human cancers, such as cancer of the prostate, breast, ovary, endometrium, colon and lung (18–20] and references therein). In addition, upregulation of FAS is an early event in tumor development; it is more pronounced in more advanced tumors and it often correlates with a poor prognosis. Although the mechanism of FAS overexpression in tumors is not completely understood, it was recently shown that growth factor-mediated signal transduction and activation of the sterol regulatory element binding protein (SREBP) pathway may play an important role in this process.[21–24] Interestingly, several studies have demonstrated that chemical FAS inhibitors, such as cerulenin and C75, inhibit growth and are cytotoxic for various tumor cell lines *in vitro*[25–27] and also have growth inhibitory effects on cancer xenografts in mice *in vivo*.[28,29]

Here, we assessed the efficacy of EGCG to block fatty acid synthesis and lipogenesis in cultured prostate cancer cells and evaluated to which extent this inhibition results in growth arrest and apoptosis.

MATERIAL AND METHODS

Cell culture

The human prostatic cancer cell line LNCaP was obtained from the American Type Culture Collection (Manassas, VA). Nonmalignant skin fibroblast cultures were kindly provided by Prof. Dr. J.J. Cassiman (Center for Human Genetics, Catholic University of Leuven, Leuven, Belgium). LNCaP cells and fibroblasts were maintained in RPMI 1640 supplemented with 10% fetal calf serum, 3 mM L-glutamine, 100 μg/ml streptomycin and 100 units/ml penicillin (Invitrogen, Carlsbad, CA). Primary cultures of normal prostatic epithelial cells were set up as described before.[30] Commercially available cultures of normal prostatic epithelial cells (PrEC 6448) were obtained from Cambrex (East Rutherford, NJ). Cell cultures were incubated at 37°C in a humidified atmosphere with 5% CO_2 and 95% air. Epigallocatechin-3-gallate (EGCG) and epicatechin (EC) (both from Sigma Chemical Co., St. Louis, MO)

Abbreviations: EC, epicatechin; EGCG, epigallocatechin-3-gallate; EGFP, enhanced green fluorescent protein; FAS, fatty acid synthase; LNCaP, lymph node carcinoma of the prostate; TLC, thin layer chromatography.

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were added to the culture medium at the indicated concentrations; cells were exposed to EGCG or EC for 24, 48 or 72 hr.

*Assay of FAS activity (*in vitro*)*

Fatty acid synthase (FAS) activity of cellular extracts was measured as previously described.[23] After 24 or 48 hr exposure to different concentrations of EGCG or EC, cells were washed with PBS, harvested by scraping in 500 μl PBS, pelleted by centrifugation and resuspended in 200 μl of a hypotonic buffer containing 1 mM DTT, 1 mM EDTA and 20 mM Tris-HCl, pH 7.5. Equal amounts of protein [40 μg and 100 μg for LNCaP cells and fibroblasts, respectively] were added to 25 μl of reaction mixture [166 mM KCl, 2.48 mM NADPH, 15 mM acetyl-CoA, 15 mM malonyl-CoA and 0.3 mM 2-^{14}C malonyl-CoA (0.35 μCi) (Perkin Elmer, Boston, MA)] and incubated for 15 min at 37°C. Reactions were stopped by addition of 1 ml ice-cold 1 N HCl/methanol (6:4, v/v). After extraction of fatty acids with petroleum benzine, incorporation of radioactivity was analyzed by scintillation counting.

Incorporation of 2-^{14}C-acetate into cellular lipids

LNCaP cells or fibroblasts were plated in 6 cm dishes, incubated overnight and treated with different doses of EGCG or EC. After 24 hr exposure to EGCG or EC, 2-^{14}C-labeled acetate (57 mCi/mmol; 2 μCi/dish; Amersham International, Aylesbury, UK) was added to the culture medium. After 4 hr incubation at 37°C, cells were washed with PBS and trypsinized. The culture medium and wash fluid were also collected. Cells were pelleted by centrifugation and resuspended in 0.8 ml PBS. Lipids were extracted using the Bligh Dyer method as previously described[31] and incorporation of 2-^{14}C-acetate into lipids was measured by scintillation counting. The results were normalized for sample protein content to avoid effects caused by differences in cell mass. To determine acetate incorporation into the different lipid fractions, lipid extracts and appropriate lipid standards were spotted on silica gel G plates (Merck, Darmstadt, Germany) and analyzed by TLC. For separation of neutral lipids plates were developed in hexane-diethyl ether-acetic acid (70:30:1, vol/vol/vol); development in chloroform-methanol-acetic acid (65:25:10, vol/vol/vol) permitted separation of phospholipids. Lipid samples and standards were visualized by autoradiography and iodine vapor, respectively, whereas a phosphorImager screen (Molecular Dynamics, Sunnyvale, CA) was used for quantification of lipid fractions, which were normalized for protein content.

Immunoblot analysis

After 48 hr exposure to different concentrations of EGCG, cells were washed with PBS and lysed in a reducing buffer containing 62.5 mM Tris pH 6.8, 2% SDS, 0.715 M 2-mercaptoethanol and 8.7% glycerol. Protein concentrations were measured using a bicinchoninic acid procedure (Pierce Biochemical Company, Rockford, IL) after precipitation with trichloroacetic acid (TCA). Equal amounts of protein were separated on NuPAGE Tris-acetate gels (Invitrogen, Carlsbad, CA) and gels were blotted onto polyvinylidene difluoride membranes (Roche, Mannheim, Germany). After blocking in a Tris-buffered saline solution with 5% nonfat dry milk, membranes were incubated with antibodies against cytokeratin 18 (Santa Cruz Biotechnology, Santa Cruz, CA) or FAS. The latter antiserum was prepared as described previously.[21] Horseradish peroxidase-conjugated secondary antibodies (Dako, Carpinteria, CA) were used for detection of immunoreactive proteins by chemiluminescence (Renaissance, New England Nuclear, Dreiech, Germany).

Proliferation/cytotoxicity assay

Cell growth and viability were analyzed with the Trypan Blue Dye Exclusion assay. At the indicated times (after 0, 24, 48 or 72 hr exposure to different concentrations of EGCG or EC), cells were washed with PBS and trypsinized. Also the culture medium and wash fluid were collected. Cells were pelleted by centrifugation and resuspended in a Trypan Blue solution and counted using a hemocytometer. The cells with and without blue dye staining were recorded as dead and alive, respectively.

Detection of apoptosis by fluorescence microscopy

Cells were plated in Lab-Tek II chamber slides (Nalge Nunc International, Naperville, IL) at a density of 1.2×10^5 LNCaP cells or 3×10^4 fibroblasts per chamber, incubated overnight and treated with EGCG or EC. After 48 hr exposure to EGCG or EC, apoptosis was evaluated. Hoechst dye 33342 (Sigma Chemical Co., St. Louis, MO) was added to the culture medium of living cells to analyze changes in nuclear morphology during apoptosis. Fragmentation of the nucleus into oligonucleosomes and chromatin condensation were detected by fluorescence microscopy using a filter for Hoechst 33342 (365 nm). Apoptosis was also determined with an Annexin V-EGFP/propidium iodide Apoptosis Detection Kit (BD Biosciences, Palo Alto, CA) according to the manufacturer's protocol. Briefly, cells were washed and incubated at room temperature in the dark during 15 min in 300 μl 1× binding buffer containing 5 μl Annexin V-EGFP and 10 μl propidium iodide. Thereafter, apoptosis was analyzed by fluorescence microscopy using a dual-filter set for EGFP (enhanced green fluorescent protein) (490 nm) and propidium iodide (560 nm).

Statistical analysis

Comparison of values was performed using one-way ANOVA. If significant differences were observed after ANOVA analysis, values were compared by a Tukey test. $p<0.05$ was considered statistically significant. Data are expressed as means ± SD. All observations were confirmed by at least 3 independent experiments.

RESULTS

EGCG is a potent inhibitor of fatty acid synthase in LNCaP cells

To study the effect of EGCG on the enzymatic activity of FAS in human prostate cancer cells, LNCaP cells were exposed to EGCG for 24 or 48 hr, cellular extracts were made and FAS activity was measured by quantification of 2-^{14}C-labeled malonyl-CoA incorporation into fatty acids *in vitro*. Equal amounts of total protein were analyzed. After 24 hr exposure to EGCG, a dose of 100 μM EGCG reduced malonyl-CoA incorporation to 52 ± 8 % of the control levels (Fig. 1*a*). A comparable inhibition by 100 μM EGCG was observed after 48 hr (Fig. 1*b*). Exposure of LNCaP cells to 150 μM EGCG for 48 hr further declined FAS activity to 22 ± 2 % of the control levels (Fig. 1*b*). Reduced FAS activity was caused by chemical inhibition of FAS enzymatic activity rather than by changes in FAS protein expression. In fact, Western blot analysis revealed that exposure of LNCaP cells to EGCG led to even higher levels of FAS protein, probably as a result of intracellular feedback control (Fig. 1*c*).

To further corroborate these findings, LNCaP cells were pretreated with EGCG for 24 hr and were then exposed to 2-^{14}C-labeled acetate, allowing the measurement of the effect of EGCG on lipid synthesis in live cells. After 4 hr, cellular lipids were extracted and acetate incorporation was quantified by scintillation counting. Increasing concentrations of EGCG gradually decreased the incorporation of 2-^{14}C-labeled acetate to 20 ± 9 % of the control levels at a dose of 150 μM (Fig. 2*a*).

To examine the impact of the reduced lipid biosynthesis in EGCG-treated LNCaP cells on the different lipid classes, lipid extracts were analyzed by TLC. Separation and quantification of the different lipid fractions revealed a dose-dependent inhibitory effect both on the synthesis of phospholipids (including phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine and sphingomyelin) and on the synthesis of triglycerides (Fig. 2*b*); 150 μM EGCG reduced phospholipid synthesis to 18 ± 10 % and triglyceride synthesis to 14 ± 7 % of the levels in control cells. Remarkably, EGCG also reduced the synthesis of cholesterol to 33 ± 4 % of the levels in control cells (Fig. 2*b*).

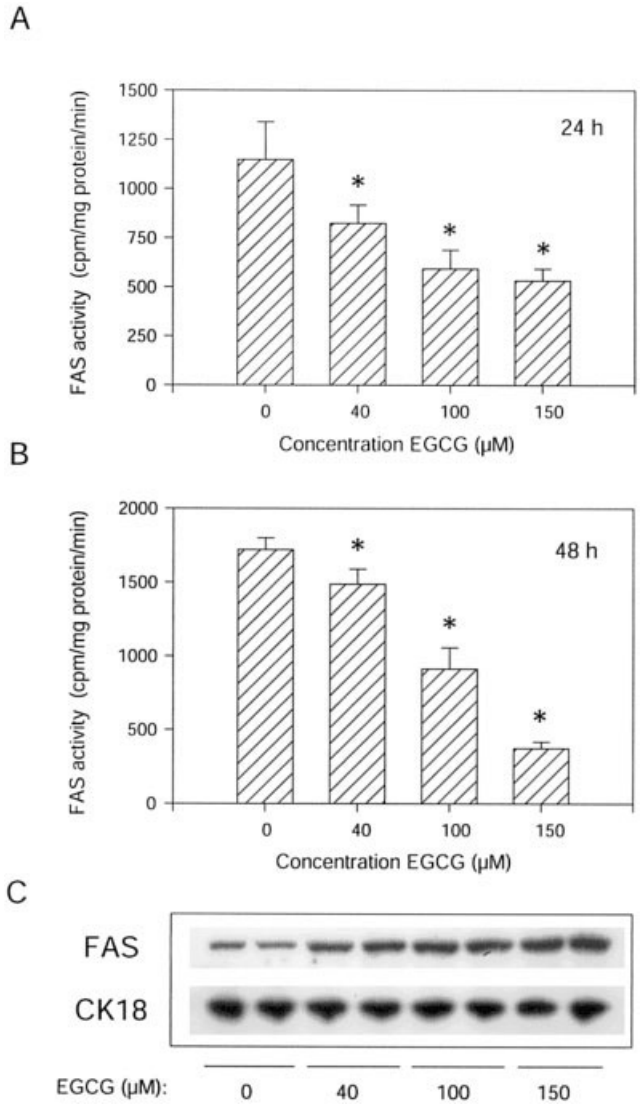

Figure 1 – Impact of EGCG on FAS activity and expression in LNCaP cells. (*a*,*b*) Cells were treated with EGCG for 24 hr (*a*) or 48 hr (*b*), protein extracts were made and FAS activity of equal amounts of total protein was measured by quantification of 2-^{14}C-labeled malonyl-CoA incorporation into fatty acids *in vitro*. Data are represented as means ± SD ($N = 4$). *Significantly different ($p<0.05$) from control cells (0 μM EGCG) by Tukey test. (*c*) Western blot analysis for FAS and cytokeratin-18 (CK18) on LNCaP cells exposed to different concentrations of EGCG for 48 hr.

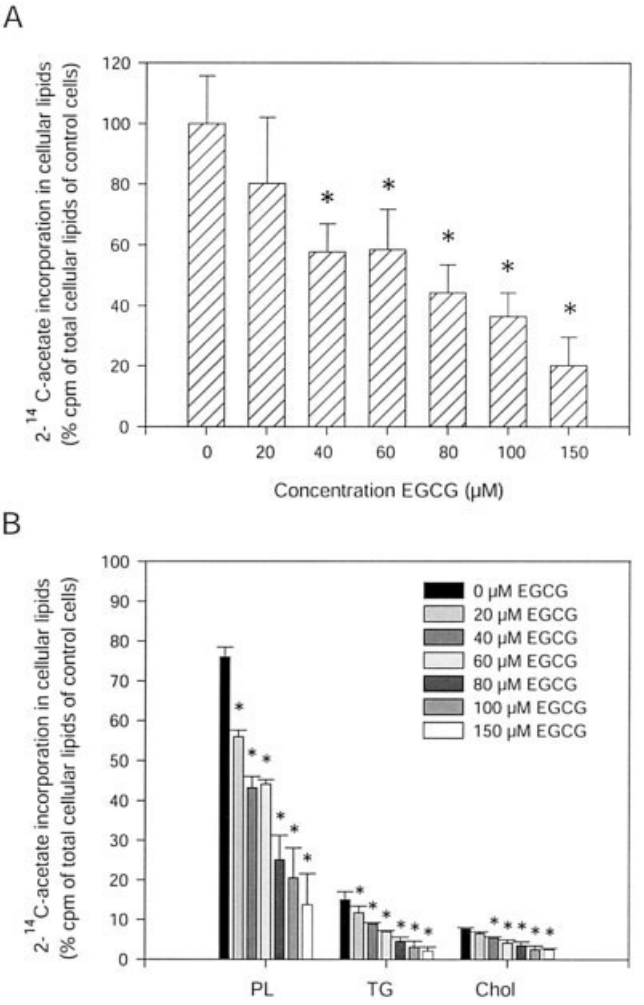

Figure 2 – Effect of EGCG on lipid biosynthesis in LNCaP cells. Cells were treated with different concentrations of EGCG for 24 hr, exposed to 2-^{14}C-labeled acetate for 4 hr and lipid extracts were prepared. (*a*) Total 2-^{14}C acetate incorporation into cellular lipids was quantified by scintillation counting. (*b*) Different lipid species were separated by TLC and ^{14}C-incorporation was quantitated; PL, phospholipids; TG, triglycerides; Chol, cholesterol. Data are represented as means ± SD ($N=6$). *Significantly different ($p<0.05$) from control cells (0 μM EGCG) by Tukey test.

Although the reason of this decline remains unclear, this inhibition could be related to the previously described ability of EGCG to inhibit *in vitro* enzymatic activity of rat squalene epoxidase, a rate-limiting enzyme in cholesterol synthesis.[32]

EGCG suppresses proliferation and induces cell death in LNCaP cells

Cell proliferation/cytotoxicity assays were performed to investigate whether EGCG-mediated inhibition of lipogenesis influenced viability of LNCaP cells. EGCG inhibited growth of LNCaP cells in a dose-dependent way (Fig. 3*a*). Whereas the cell number of control cultures (0 μM EGCG) increased by 7-fold in 72 hr, the number of viable cells remained unchanged after treatment with 150 μM EGCG. Trypan blue staining also revealed cell death of LNCaP cells, particularly at the highest concentrations of EGCG used (Fig. 3*b*). Growth inhibition and cell death were most pronounced at EGCG concentrations that also inhibited FAS activity and 2-^{14}C-labeled acetate incorporation by 50% or more (see Figs. 1*a*,*b* and 2*a*,*b*). A similar level of FAS inhibition has previously been related to growth arrest and cell death using well known FAS inhibitors such as cerulenin and C75.[25,26,29]

FAS inhibition by EGCG results in apoptosis of LNCaP cells

To examine the mechanism by which EGCG induces cell death in LNCaP cells, cells were stained using both Hoechst 33342 and an apoptosis detection kit based on the use of Annexin V-EGFP and propidium iodide (Fig. 4). Hoechst staining of EGCG-treated LNCaP cells (100 μM for 48 hr) revealed nuclear fragmentation and chromatin condensation, typical characteristics of apoptosis. The presence of apoptosis was confirmed by staining with propidium iodide (a membrane-impermeable dye) and Annexin V-EGFP (a protein that binds with high affinity to the phospholipid phosphatidylserine). In normal cells, phosphatidylserine is located exclusively in the inner side of the cellular membrane, whereas in apoptotic cells

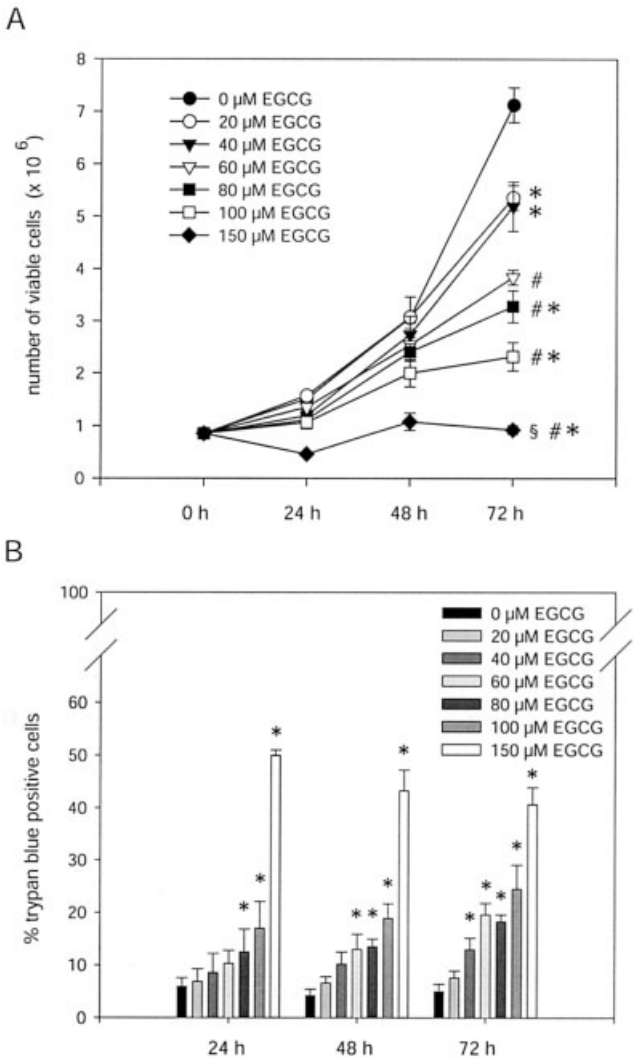

FIGURE 3 – Impact of EGCG on LNCaP cell number and viability. LNCaP cells were treated with different concentrations of EGCG. At the indicated time points cells were stained with Trypan Blue and viable (*a*) and dead cells (*b*) were counted. Data are represented as means ± SD (N=4). (*a*) Section sign, number sign, asterisk, significantly different from control cells (0 µM EGCG) after 24 hr (section sign),48 hr (number sign), and 72 hr (asterisk), respectively, by Tukey test. (*b*) Asterisk, Significantly different ($p<0.05$) from control cells (0 µM EGCG) by Tukey test.

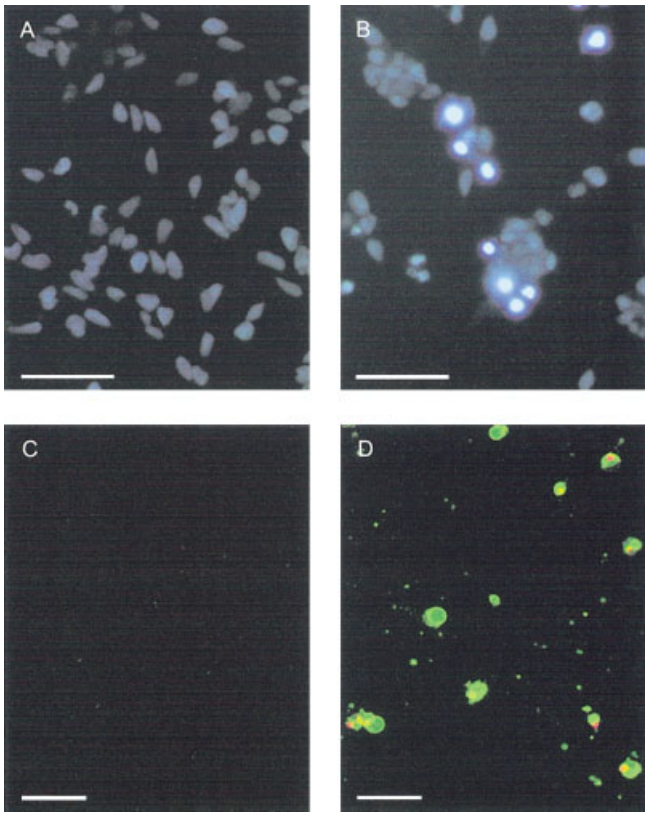

FIGURE 4 – EGCG-induced apoptosis of LNCaP cells. LNCaP cells were treated with 0 µM (*a*,*c*) or 100 µM EGCG (*b*,*d*) for 48 hr. Afterwards, cells were stained with Hoechst 33342 (*a*,*b*) or with Annexin V-EGFP and propidium iodide (*c*,*d*), and analyzed by fluorescence microscopy. Bar indicates 50 µm.

it is translocated to the outer membrane leaflet. Many LNCaP cells, exposed to EGCG, stained positive for Annexin V-EGFP but not for propidium iodide (Fig. 4*d*), a pattern reflecting apoptosis. Some cells stained positive for both Annexin V-EGFP and propidium iodide (Fig. 4*d*), a pattern reflecting membrane disruption that is characteristic for later stages of apoptosis and for necrosis. Taken together, these data indicate that EGCG-induced cell death is at least in part the result of apoptosis.

EC, a tea polyphenol that has no inhibitory effect on FAS, does not influence proliferation or viability of LNCaP cells

To further illustrate the correlation between EGCG-induced FAS inhibition and LNCaP cell death, LNCaP cells were also exposed to another closely related green tea polyphenolic compound, more particularly epicatechin (EC). In contrast with EGCG, similar doses of EC did not inhibit FAS in LNCaP cells, as revealed by FAS activity measurements and 2-^{14}C-acetate incorporation assays. Both *in vitro* enzymatic activity of FAS (Fig. 5*a*) and lipid biosynthesis (Fig. 5*b*) were similar in EC-treated and control LNCaP cells, even at the highest EC dose used (150 µM). Interestingly, cell proliferation assays (Fig. 5*c*), cytotoxicity assays (Fig. 5*d*) and Hoechst stainings (not shown) revealed that EC also had no effect on LNCaP cell growth or viability. These findings demonstrate that a catechin that has no inhibitory effect on FAS (such as EC), does not induce prostate cancer cell death.

EGCG induces apoptosis in LNCaP prostate cancer cells but not in normal human fibroblasts

To analyze the selectivity of the EGCG-induced apoptosis for FAS-overexpressing cancer cells, we also studied the effect of EGCG on nonmalignant cells with low FAS expression. Since both our own[30] and commercially available cultures of normal prostatic epithelial cells, which normally have low FAS protein levels *in vivo*,[18] exhibited high levels of FAS expression when cultured *in vitro*, we turned to nonmalignant human skin fibroblast cultures as a model of low FAS expressing cells. Baseline FAS activity levels were 15 to 20 times lower in fibroblasts than in LNCaP cells (Figs. 1*a*,*b* and 6*a*). EGCG caused a further decrease of FAS activity (Fig. 6*a*) and induced a dose-dependent inhibition of the proliferation rate of normal fibroblasts comparable to that observed in LNCaP cells (Fig. 6*b*). However, in contrast with LNCaP cells, trypan blue dye exclusion assays showed that EGCG did not induce cell death in normal fibroblasts (Fig. 6*c*), even not at a concentration of 150 µM EGCG, a concentration that caused nearly 50% of apoptosis in LNCaP cells. In addition, Hoechst staining and Annexin V-EGFP/propidium iodide staining also

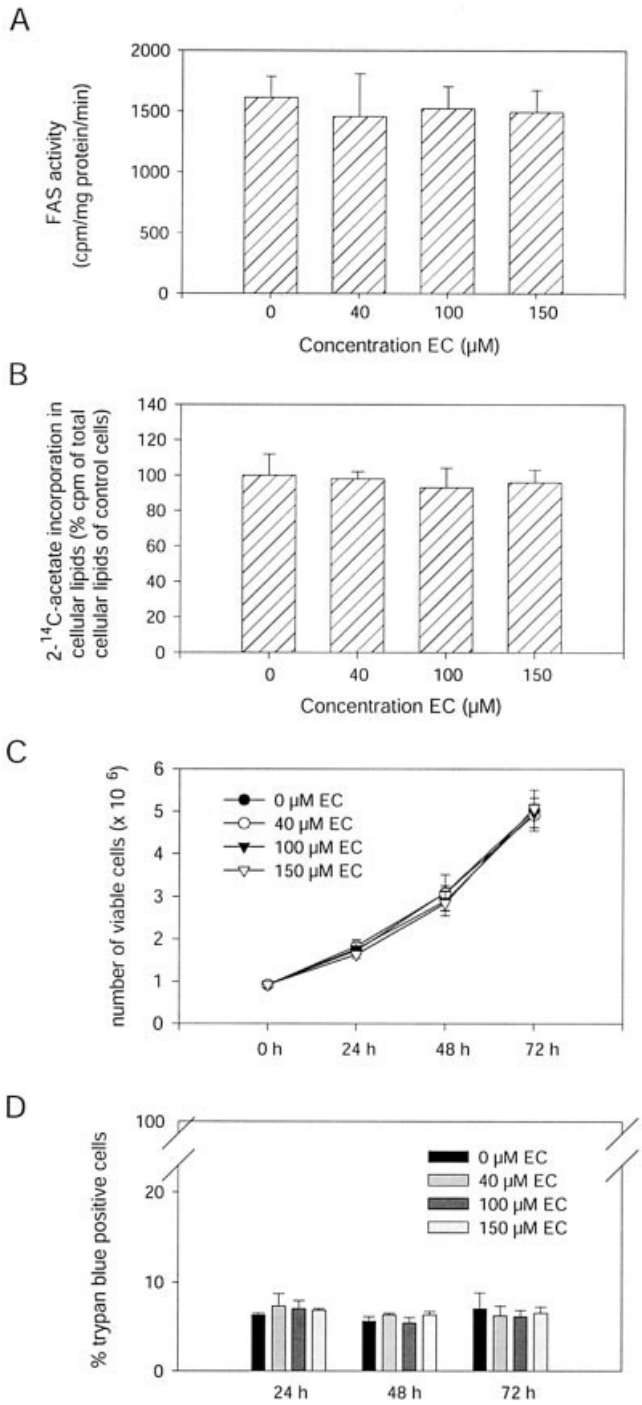

FIGURE 5 – Effect of EC on FAS activity, lipid synthesis, cell growth and viability of LNCaP cells. (*a*) FAS activity of extracts from LNCaP cells treated with different concentrations of EC for 48 hr, as measured by quantification of 2-^{14}C-labeled malonyl-CoA incorporation into fatty acids *in vitro*. (*b*) Lipid biosynthesis of LNCaP cells treated with different concentrations of EC for 24 hr, as analyzed by quantifying 2-^{14}C acetate incorporation into cellular lipids. (*c*,*d*) Proliferation (*c*) and cell death (*d*) of LNCaP cells treated with different concentrations of EC, as revealed by counting the number of viable and dead cells using the Trypan Blue Dye exclusion assay. Data are represented as means ± SD (N=4). No significant (P<0.05) differences were observed in the EC-treated groups when compared to the control group (0 μM EC) by Tukey test.

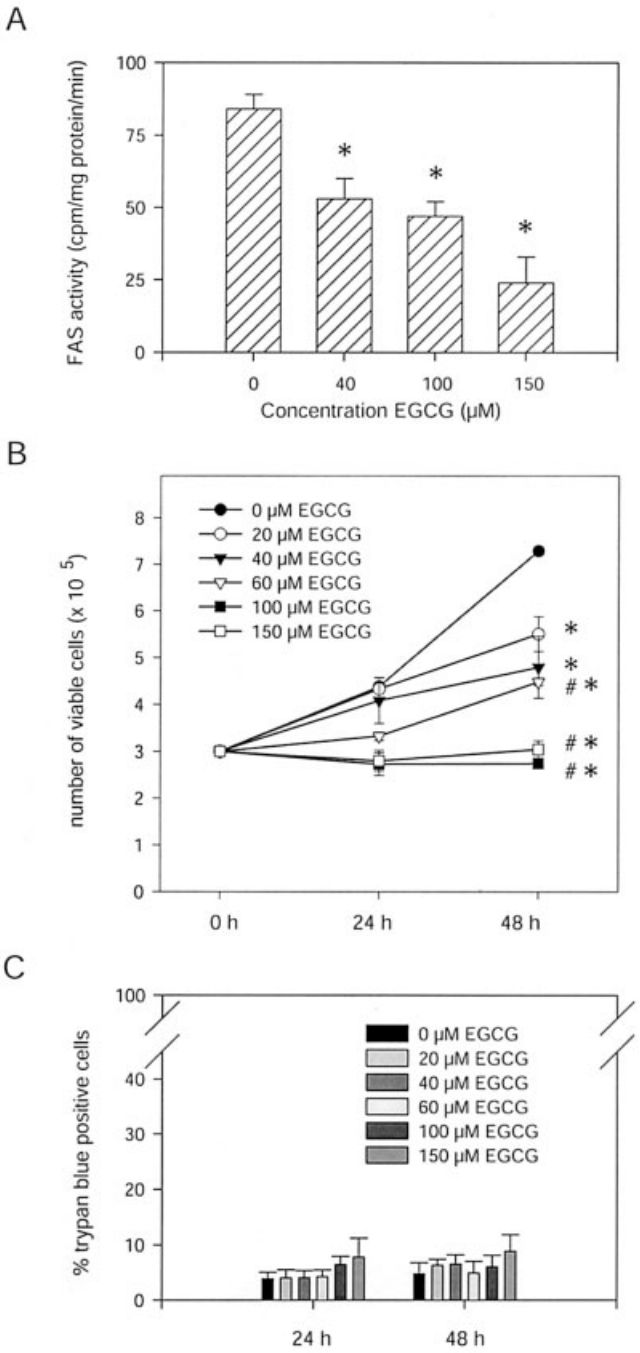

FIGURE 6 – Impact of EGCG on normal human fibroblasts. (*a*) FAS activity of extracts from fibroblasts treated with different concentrations of EGCG for 48 hr, as measured by quantification of 2-^{14}C-labeled malonyl-CoA incorporation into fatty acids *in vitro*. (*b*,*c*) Proliferation (*b*) and cell death (*c*) of fibroblasts treated with different concentrations of EGCG, as revealed by counting the number of viable and dead cells using the Trypan Blue Dye exclusion assay. Data are represented as means ± SD (N=4). (*a*,*c*) *Significantly different (p<0.05) from control (0 μM EGCG) by Tukey test. (*b*) Number sign, asterisk, significantly different (p<0.05) from control cells (0 μM EGCG) after 24 hr (number sign) and 48 hr (asterisk), respectively, by Tukey test.

failed to reveal any signs of apoptosis in EGCG-treated fibroblasts (data not shown). These data indicate that cultured fibroblasts have markedly lower levels of FAS activity and are far less sensitive to EGCG-induced cell death.

DISCUSSION

EGCG has recently been shown to be a potent *in vitro* inhibitor of the FAS-enzyme isolated from chicken liver.[16] In the present article, we show that EGCG also inhibits FAS activity in cultured human prostate tumor cells and that this inhibition is paralleled by a marked reduction in cell proliferation and by a cancer cell-specific increase in cell death that is largely related to apoptosis.

The EGCG-induced inhibition of FAS was evident both in cell extracts and in intact cells. In extracts of LNCaP cells pre-treated with EGCG, FAS activity (measured as incorporation of 2-^{14}C-malonyl-CoA into fatty acids) was reduced in a dose-dependent way. Fifty percent inhibition was observed at a concentration of 50–100 μM (a concentration comparable to the IC50 value of 52 μM observed for the reversible fast-binding inhibition of FAS derived from chicken liver[16]) and at the highest concentration studied (150 μM EGCG) FAS activity was reduced to 22% of the control levels. Interestingly, this inhibition is accompanied by a marked increase in FAS protein levels, suggesting some form of intracellular feedback. In intact cells the decrease in FAS activity was evidenced by a decrease in the incorporation of 2-^{14}C-acetate in cellular lipids and more particularly by a decrease in labeling of lipid fractions that are rich in fatty acids such as phospholipids (decrease to 18% of control levels) and triglycerides (decrease to 14% of control levels). Surprisingly, cholesterol synthesis was also decreased (to 33% of control levels) by EGCG, although not as markedly as phospholipid and triglyceride synthesis. A similar decrease in cholesterol synthesis has also been reported for other FAS-inhibitors such as cerulenin.[33,34] Since FAS is not directly involved in cholesterol synthesis, such a decrease might be the result of secondary changes in intracellular lipid homeostasis. Recent data obtained in our laboratory, however, indicate that a selective knockdown of FAS expression using RNA interference technology does not affect cholesterol synthesis (unpublished results). Accordingly it seems more likely that the effects on cholesterol reflect an alternative site of EGCG action, such as the previously reported inhibition of squalene epoxidase, a rate-limiting enzyme along the cholesterol synthesis pathway.[32]

In parallel with its effects on FAS activity and lipid synthesis EGCG decreased proliferation of LNCaP tumor cells and induced apoptosis. These effects are reminiscent of the growth inhibitory and cytotoxic effects observed with chemical FAS inhibitors such as cerulenin and C75 in a variety of tumor cell lines (including LNCaP) and in xenograft models.[25–29] Although alternative sites of EGCG action in tumor cells can certainly not be excluded, the dose-response curves reflecting FAS inhibition and those reflecting inhibition of proliferation show a striking parallelism. Moreover, in contrast with EGCG, epicatechin (EC), another closely related polyphenolic compound that is also present in green tea but does not inhibit FAS, has no effect on LNCaP tumor cell growth or viability. Furthermore, the antiproliferative effects of EGCG are observed at a level of inhibition of FAS activity comparable to that required to reach similar effects with cerulenin and C75 ([25,26,29] and unpublished results). Cell death became prominent at concentrations of EGCG exceeding 40 μM and reached values of 40–50% at a concentration of 150 μM. Although some contribution of necrosis cannot be excluded, the majority of cells displayed features that point to apoptosis (nuclear condensation and fragmentation, staining for Annexin-V in the absence of staining for propidium iodide). Taken together our data indicate that EGCG inhibits FAS activity in LNCaP tumor cells and suggest that this inhibitory effect may be responsible for the observed antiproliferative and cytotoxic properties.

To explore whether EGCG has potential as a chemotherapeutic compound selectively attacking cancer cells, we also studied the effects on nontumoral fibroblasts. As the vast majority of normal tissues, such fibroblasts display a very low level of FAS activity (10–20 times lower than that observed in LNCaP cells). These levels were efficiently inhibited by EGCG and again this inhibition was accompanied by dose-dependent antiproliferative effects. In contrast with LNCaP tumor cells, however, cell death was not significantly increased even at the highest levels of EGCG tested and apoptosis was not observed. The pathways by which FAS inhibitors decrease proliferation and induce apoptosis remain poorly understood but at least 2 elements may be involved. First, inhibition of fatty acid synthesis may limit membrane synthesis required for cell growth and division, and, second, inhibition of FAS may also result in the accumulation of precursors and some of these precursors such as malonyl-CoA may be toxic for the cell.[29,35] Taking into account that FAS activity is markedly increased in a variety of epithelial tumors[18–20] and that this increase is only part of a more general stimulation of lipogenesis, also involving enzymes catalyzing early steps of fatty acid synthesis,[22] it may be anticipated that accumulation of toxic precursors will be more important in tumor cells. In this context, it is tempting to speculate that the selective cytotoxicity of EGCG to tumor cells is related to the accumulation of such toxic precursors. However, it can not be excluded that EGCG, in addition to the induction of LNCaP cell death *via* FAS inhibition, may also influence LNCaP cell viability by other effects. EGCG has been previously shown to inhibit tumor-promoting factors such as matrix metalloproteinases, vascular endothelial growth factor and sex steroids[13,14,36] and to cause inhibition of Bcl-XL phosphorylation (which is associated with prostate cancer cell apoptosis).[37]

The question should be asked whether the above described ability of EGCG to reduce FAS activity contributes to the epidemiologically observed inverse correlation between the consumption of green tea and the risk of developing various cancers (including cancer of the breast, prostate, stomach, esophagus, colon and pancreas)[2–6] and to the decrease in tumor size noticed in EGCG-treated mice or rats,[3,10–12] A definitive answer to this question will require additional studies but a number of points merit consideration. First, *in vivo* administration of EGCG reduces food intake and increases energy expenditure,[36,38] which are remarkable effects that are also observed after *in vivo* administration of other FAS inhibitors such as C75 and cerulenin.[39,40] Although the pathogenesis of these effects is not entirely understood, studies with C75 and cerulenin suggest that FAS inhibition is an essential element involved.[39,41] Second, although the plasma concentrations of EGCG after a single oral administration seem to be in the 0.2 to 4.4 μM range in humans[42–44] and the 1–14 μM range in rats[45] (and accordingly lower than the 50–100 μM concentrations required in the here reported culture experiments), considerably higher concentrations (50–560 nmol/g) have been described in tissues.[45] Moreover, a second administration of EGCG has been shown to result in plasma and organ levels 4–6 times higher than those observed after a single dose,[46] indicating that frequent consumption of green tea or EGCG may significantly increase EGCG concentrations in plasma and organs. Third, FAS inhibition by EGCG is composed of reversible fast-binding inhibition and irreversible slow binding inactivation.[16] Although the present data suggest that EGCG initiates a compensatory feedback reaction that results in higher levels of FAS enzyme, it is conceivable that long-term exposure even to low doses of EGCG might favor irreversible inhibition.

It may be concluded that EGCG-mediated FAS inhibition is undoubtedly one of the mechanisms that merit consideration when trying to explain the cancer chemopreventive and chemotherapeutic potential of this green tea polyphenol. The relatively low toxicity of this compound, the multiple alternative pathways by which it may favorably affect tumor development (such as interference with the production of sex steroids, insulin, insulin-like growth factor-1 and vascular endothelial growth factor,[13,14,36] and inhibition of matrix metalloproteinases,[13] protein tyrosine kinases[3,13] and Bcl-XL phosphorylation[37]) and its beneficial effects on major health risk factors such as atherosclerosis, hypertension, coronary heart disease and osteoporosis [3,47] make EGCG an attractive target for further pharmacochemical studies.

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